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Research Article

Hyperglycemia Does Not Affect Iron Mediated Toxicity of Cultured Endothelial and Renal Tubular Epithelial Cells: Influence of L-Carnosine

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Iron has been suggested to affect the clinical course of type 2 diabetes (T2DM) as accompanying increased intracellular iron accumulation may provide an alternative source for reactive oxygen species (ROS). Although carnosine has proven its therapeutic efficacy in rodent models of T2DM, little is known about its efficacy to protect cells from iron toxicity. We sought to assess if high glucose (HG) exposure makes cultured human umbilical vein endothelial cells (HUVECs) and renal proximal tubular epithelial cells (PTECs) more susceptible to metal induced toxicity and if this is ameliorated by L-carnosine. HUVECs and PTECs, cultured under normal glucose (5 mM, NG) or HG (30 mM), were challenged for 24 h with FeCl₃. Cell viability was not impaired under HG conditions nor did HG increase susceptibility to FeCl₃. HG did not change the expression of divalent metal transporter 1 (DMT1), ferroportin (IREG), and transferrin receptor protein 1 (TFRC). Irrespective of glucose concentrations L-carnosine prevented toxicity in a dose-dependent manner, only if it was present during the FeCl₃ challenge. Hence our study indicates that iron induced cytotoxicity is not enhanced under HG conditions. L-Carnosine displayed a strong protective effect, most likely by chelation of iron mediated toxicity.

1. Introduction

High body iron levels are associated with increased levels of oxidative stress that may elevate the risk of T2DM [1–7]. Indeed, epidemiological studies have indicated a positive association between high body iron stores and the risk of T2DM and of other insulin resistant states such as metabolic syndrome, gestational diabetes, and polycystic ovarian syndrome [8–11]. High ferritin levels in T2DM are closely related to the development of diabetic vascular complications, possibly through the interaction with vascular endothelial growth factor (VEGF) [12] or through endothelial dysfunction [13]. It has also been suggested that an increased

transferrin excretion in diabetic patients with microalbuminuria may contribute to tubulointerstitial injuries [14] as a consequence of transferrin reabsorption by PTECs. This in turn increases intracellular iron concentrations leading to oxidative damage of the tubular cells [7]. The relationship between iron metabolism and T2DM seems to be bidirectional since iron affects glucose metabolism via its deleterious effect on pancreatic β -cells, and glucose metabolism impinges on several iron metabolic pathways [4]. However, studies performed in the Belgrade rat, which carries a mutation in the iron transporter DMT1, demonstrate that these rats displayed normal glycemic control and insulin signaling and secretion despite high levels of nonheme iron [15].

It is noteworthy to mention that a number of structures arising secondary to protein glycation might bind transition metals such as iron and copper. The bound metal retains redox active and participates in catalytic oxidation reactions [16]. Iron depletion has been demonstrated to be beneficial in coronary artery responses, endothelial dysfunction, insulin secretion, insulin action, and metabolic control in T2DM.

Carnosine has antioxidant properties and is efficient in the treatment of chemically induced inflammatory lesions in animals [17, 18], fatty acid mediated lipid peroxidation [19], and numerous disease models in which oxidative stress plays a pivotal role [20, 21]. Also in diabetic models oral carnosine supplementation has proven to ameliorate apoptosis of glomerular cells [22], to prevent alterations in carnosine metabolism [23], to improve hyperglycemia [24], and to ameliorate dyslipidemia [25]. These changes are associated with improved renal function, albeit that renal pathology in such models is mostly relatively minor. The mechanism by which carnosine exerts its beneficial effects is multifactorial including chelation of transition metal ions [26, 27], inhibition of advanced glycation end products (AGEs), and inhibition of advanced lipoxidation end products (ALEs) [28].

In keeping with the protective effect of carnosine in diabetic models and the relevance of increased iron stores in T2DM patients, we sought to address if high glucose exposure makes cultured human endothelial cells (ECs) and PTECs more susceptible to iron toxicity and if this is mitigated in the presence of carnosine.

2. Methods and Materials

2.1. Cell Culture. HUVECs were isolated from fresh human umbilical cords. The cells were cultured on 1% gelatin (Fluka, Neu-Ulm, Germany) coated culture flasks at 37°C and 5% CO₂ in endothelial cell growth medium (Provitro, Berlin, Germany) containing 50 ng/mL amphotericin B together with 50 µg/mL gentamicin and 2% fetal calf serum (FCS). PTECs were isolated from human kidney biopsy specimen and cultured in DMEM/F-12 medium (DMEM/F-12, GlutaMAX, Invitrogen, Karlsruhe, Germany) supplemented with 36 ng/mL hydrocortisone, 5 µg/mL transferrin, 5 ng/mL sodium selenite, 4 pg/mL triiodo-L-thyronine, 5 µg/mL insulin, and 10 ng/mL EGF at 37°C and 8% CO₂. The cells were grown on a 0.01% collagen (Sigma, Munich, Germany) coating to which FCS was added. All experiments involving HUVECs and PTECs were performed in passages 3 to 6.

2.2. Cell Viability Assay. HUVECs or PTECs were seeded in 96-well plates and cultured for 2 consecutive days under normal glucose (5 mM D-glucose) or high glucose (30 mM D-glucose) conditions. Hereafter the cells were challenged for 24 h with different concentrations of FeCl₃ (Sigma, Munich, Germany) or ZnCl₂ (Sigma, Munich, Germany) either in the presence or in the absence of L-carnosine (Sigma, Munich, Germany). In some experiments, the cells were pretreated for 2 days with L-carnosine and subsequently challenged on day 3 with FeCl₃ in the absence of L-carnosine. Hereafter

cytotoxicity was assessed by MTT assay as described in [29]. Cell viability was calculated as percentage relative to cells that were not challenged with FeCl₃. In each assay and for each condition at least 6 replicates were used. LC₅₀ curves were based on at least 4 independent experiments.

2.3. TUNEL Assay. DNA damage of HUVECs and PTECs was assessed after treatment by TUNEL assay according to the manufacture's protocol using in situ cell death detection kit fluorescein (Roche, Mannheim, Germany). The excitation wavelength of 488 nm was applied for fluorescence microscope, and the detection wavelength was FITC Green (515–565 nm).

2.4. RNA Isolation and qPCR. Total RNA from HUVECs or PTECs was extracted and cDNA was synthesized as described in [30]. qPCR was performed using TaqMan universal PCR master mix (Applied Biosystems, Darmstadt, Germany) on an ABI-Prism 7700 detection system. The TaqMan assays were performed for DMT1 (material number Hs00167206.m1), TFRC (material number Hs00951083.m1), and GAPDH (material number Hs02758991.g1) for normalization.

2.5. FACS Analysis. The expression of the TFRC on HUVECs and PTECs was assessed by indirect immune-fluorescence staining and FACS analysis. To this end, cells were incubated for 30 min at 4°C with monoclonal antibodies directed against TFRC (Abcam, Cambridge, UK). Hereafter the cells were thoroughly washed and the 2nd antibodies were conjugated to FITC. The cells were washed twice to remove unbound antibodies and were finally resuspended in 300 µL of Cell Wash (BD Biosciences). Analysis was performed on a FACS Calibur flow cytometer (BD Biosciences) and data were analyzed using WinMDI version 2.8 software.

2.6. Western Blot. For detection of DMT1 or carnosinase-1 (CN-1) expressed in HUVECs and PTECs, gel electrophoresis and western blotting were performed. All samples (20 µg of total protein) were 1:1 diluted in Laemmli buffer (Bio-Rad, Munich, Germany) and boiled for 5 min before loading on a SDS-PAGE gel. After electrophoresis proteins were transferred to PVDF membranes (Roche, Mannheim, Germany) by semidry blotting as described before [31]. Anti-DMT1 (Abcam, Cambridge, UK) or anti-CNDP1 (Sigma, Munich, Germany) was used as the first antibody afterwards.

2.7. Carnosinase Activity. Carnosine was incubated with 300 µM FeCl₃ for 30 min prior to the initiation of the assay. CNDP1 recombinant human protein (2 ng/µL) (Life Technology, Darmstadt, Germany) was hereafter added to either carnosine or carnosine-FeCl₃ mix. Carnosinase activity was afterwards determined according to the method described by Teufel et al. [32].

2.8. Lentivirus Transduction. CN-1 cDNA was constructed as described before [24] from IMAGE clone accession number BX094414 and then cloned into HIV based lentivirus vector pPM337. Viruses were produced in HEK293 cells using

pCMV891 and pMD.G plasmids. Supernatants of HEK293 cells were collected and concentrated as virus solution. For transduction, HUVECs were incubated in 1:100 diluted virus solutions for 48 hours hereafter. Cell lysates were obtained by a freeze-and-thaw cycle in liquid nitrogen. Western blotting of 20 μ g total protein from HUVECs supernatant and cell lysates was performed to confirm CN-1 expression. Transduced and wild type HUVECs were challenged with iron in the presence or absence of carnosine.

2.9. Statistics. Data between two groups were evaluated by *t*-test, and differences for multiple groups were assessed by one-way ANOVA. Significance was defined according to a *p* < 0.05.

3. Results

3.1. Susceptibility of Endothelial and Tubular Epithelial Cells to Transition Metals. To assess susceptibility of HUVECs and PTECs to transition metal mediated toxicity cells were challenged for 24 h with different concentrations of FeCl₃ or ZnCl₂. While HUVECs were equally susceptible to FeCl₃ or ZnCl₂ (LC₅₀: ~0.1 mM for both transition metals), PTECs were more susceptible to ZnCl₂ (LC₅₀: ~0.1 mM) as compared to FeCl₃ (LC₅₀: ~5 mM). Susceptibility to FeCl₃ was significantly higher in HUVECs than in PTECs (Figures 1(a)–1(c)). Since HUVECs medium contains 2% FCS while PTECs are cultured in serum-free culture medium supplemented with transferrin, we tested if this might explain the difference in susceptibility between HUVECs and PTECs. Even if HUVECs were challenged in PTECs medium in the absence of transferrin the cells were highly susceptible to FeCl₃ and no increase in susceptibility was observed with increasing transferrin concentrations (Figure 1(d)). PTECs became also susceptible to lower concentration of FeCl₃ (400 μ M) if they were challenged over a longer period of time (Figure 1(e)). Transition metal mediated toxicity was associated with DNA damage as evidenced by an increased number of TUNEL positive cells (Figure 1(f)).

3.2. Carnosine Prevents Iron Mediated Toxicity Regardless of the Presence of High Glucose. We next assessed if HG conditions make the cells more vulnerable to FeCl₃ mediated toxicity. Cell viability of HUVECs and PTECs was not impaired under HG conditions (3 days of culture) nor was HG associated with increased susceptibility to FeCl₃. When the cells were challenged in the presence of carnosine, toxicity was strongly abrogated under NG and HG conditions (Figures 2(a) and 3(b)). For both HUVECs and PTECs the protective effect of carnosine revealed a dose-dependent relation (Figures 2(c) and 2(d)). Protection only occurred when carnosine was present during the FeCl₃ challenge but not when carnosine was used as pretreatment only (Figures 2(e) and 2(f)). Similar to carnosine also deferoxamine (DFO) was able to protect HUVECs (Figure 2(g)) and PTECs (data not shown) against iron mediated toxicity, albeit that at higher concentrations of FeCl₃ the protection by 100 μ M of DFO started to wean off. Higher concentrations of DFO were not

used as these concentrations negatively affected cell viability, independent of FeCl₃.

3.3. Ectopic CN-1 Expression in HUVECs Does Not Abrogate Protection by Carnosine. To assess if aberrant overexpression of serum carnosinase would mitigate protection against FeCl₃ mediated toxicity, HUVECs were transduced with *CNDPI* cDNA by lentivirus. CN-1 expression was detected in both cell lysates and supernatants of *CNDPI* transduced HUVECs but not in wild type HUVECs (data not shown). Susceptibility to FeCl₃ was not changed upon *CNDPI* transduction nor was the protective effect of carnosine abrogated (Figure 3(a)). To exclude that protection by carnosine was at large mediated via its constituent amino acids we performed similar experiments with β -alanine or L-histidine. Although L-histidine showed a minimal but significant level of protection when iron concentration was at 150 μ M, protection was by far not comparable to that of carnosine (Figure 3(b)). Nevertheless, the minor protective effect of L-histidine was diminished when iron concentration was at 300 μ M. Carnosinase protein and activity were found in both the cell lysate and supernatants of *CNDPI* transduced HUVECs but were significantly lower as compared to human serum. We also tested whether carnosine-iron complex would be resistant to the hydrolysis of carnosinase. In this setting, carnosine was incubated with 300 μ M FeCl₃ for 30 min prior to the initiation of carnosinase activity measurement. Indeed carnosinase activity was hardly detectable when iron was added (Figure 3(c)), suggesting either a higher resistance of iron complexed carnosine to hydrolysis by serum carnosinase or direct inhibition of serum carnosinase by iron.

3.4. Influence of Iron and Carnosine on Iron Transporters. The expression of two major iron transporters, that is, DMT1 and TFRC, was investigated at mRNA and protein level in both HUVECs and PTECs. To avoid toxicity, HUVECs and PTECs were cultured for 24 h in the presence of low iron concentration (60 μ M). While in HUVECs DMT1 mRNA was strongly downregulated by iron and carnosine, in PTECs the effect was small (Figure 4(a)). TFRC mRNA expression was downregulated in both HUVECs and PTECs by iron and carnosine (Figure 4(b)). Although downregulation of DMT1 mRNA was observed in the presence of iron and carnosine this was not reflected by a decrease in DMT1 protein expression (Figure 4(c)). In HUVECs TFRC protein expression was slightly decreased by iron, but not by carnosine. In contrast, both iron and carnosine decreased the expression of TFRC on PTECs (Table 1).

4. Discussion

A recent trial to assess chelation of transition metals by EDTA, that is, the Trial to Assess Chelation Therapy (TACT) study [33], has provided evidence that intravenous chelation therapy may reduce the risk of mortality and vascular events in diabetics who had previously experienced a myocardial infarction, whereas no benefit was observed in nondiabetics. Also a number of studies have indicated that transition

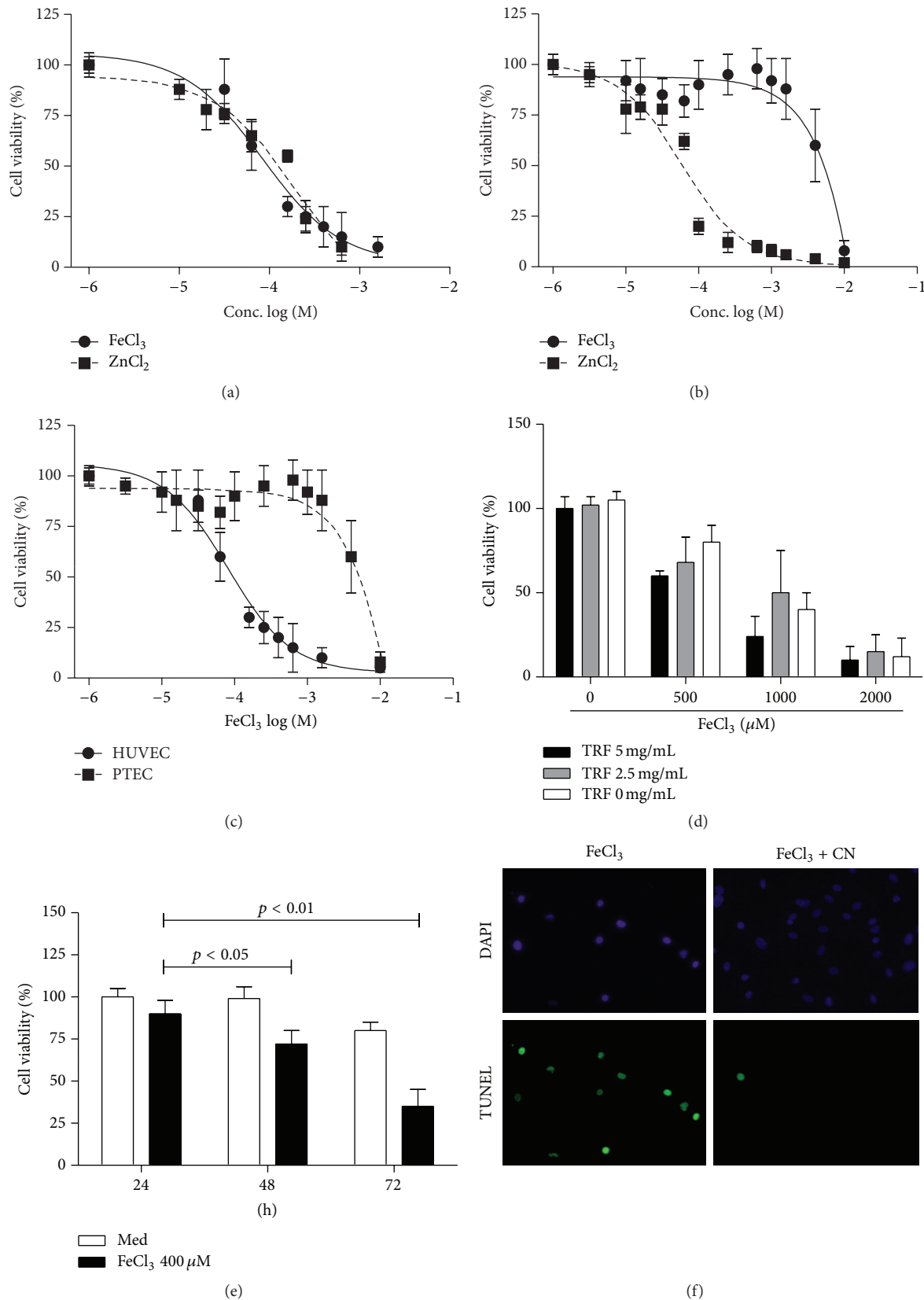


FIGURE 1: Iron and zinc mediated toxicity in HUVECs (a) and PTECs (b). PTECs were more tolerant to iron toxicity as compared to HUVECs (c). Iron mediated toxicity was not largely influenced by transferrin (TRF) (d). Although PTECs were less susceptible to iron mediated toxicity, toxicity increased upon longer exposure (e). Iron toxicity was associated with an increased number of TUNEL positive cells, which was significantly abrogated by L-carnosine (f).

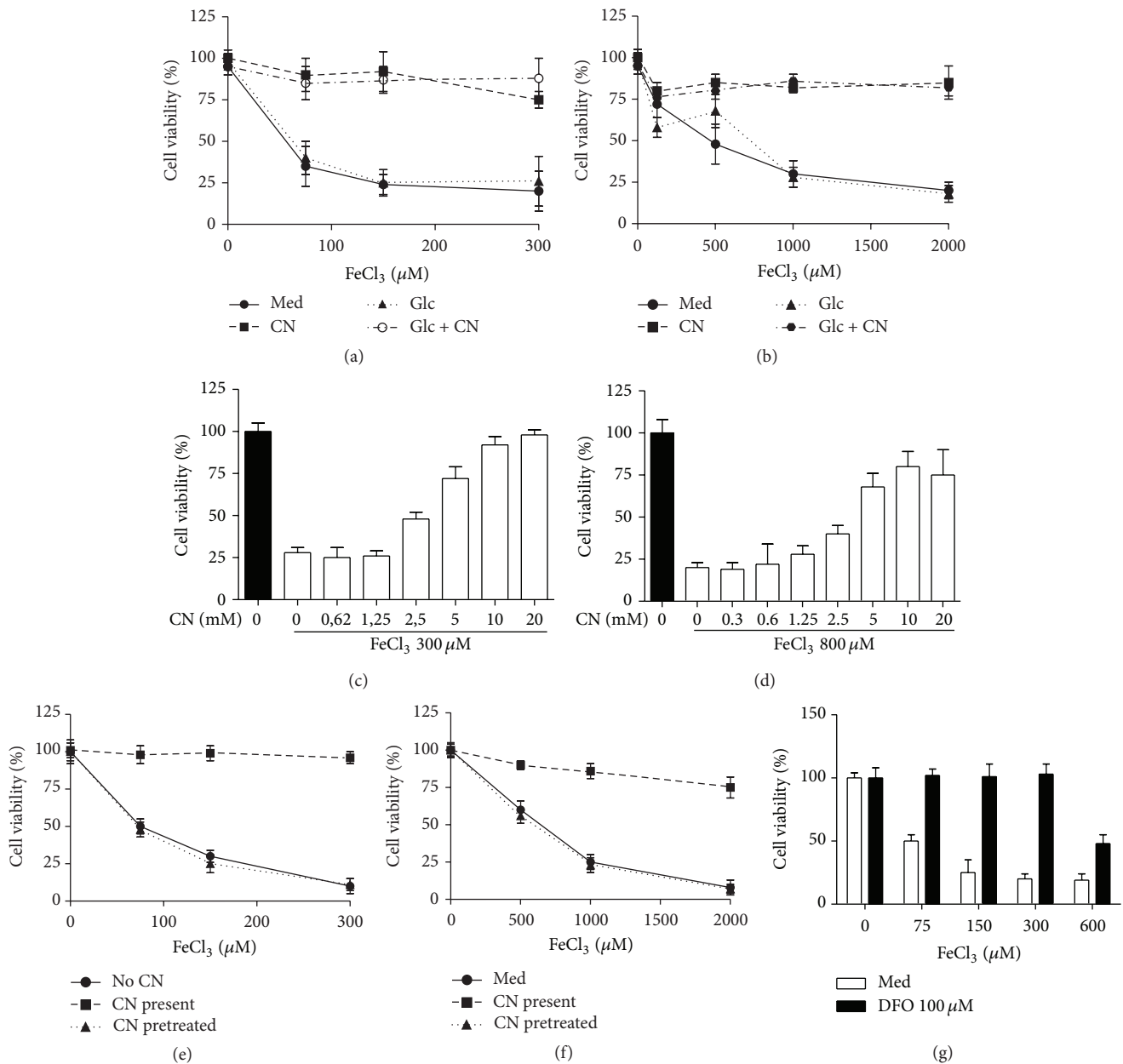


FIGURE 2: Influence of high glucose and carnosine on iron mediated toxicity in HUVECs (a, c, and e) and PTECs (b, d, and f). High glucose concentration neither increased cell susceptibility to iron nor disrupted the protective effect of carnosine on iron toxicity in HUVECs (a) and PTECs (b). The protective effect of carnosine showed a clear dose-dependent relation in both HUVECs (c) and PTECs (d). Carnosine was only protective when presented during iron challenge but not when cells were pretreated (e and f). Like carnosine also the iron chelator DFO prevented iron mediated toxicity in HUVEC when iron concentration was less than 600 μM (g). Med: medium, CN: carnosine, Glc: high glucose, and Glc + CN: high glucose + carnosine.

metals, in particularly iron, may have a large impact on the development of diabetes and diabetic complications [34–36]. Since serum carnosinase, encoded by the *CNDPI* gene, has been suggested to be implicated in susceptibility to diabetic nephropathy, the present study was carried out to assess if carnosine, the natural substrate of serum carnosinase, is able to mitigate iron mediated toxicity in endothelial and proximal tubular cells. The main findings of our study are as follows. Firstly, HG does not make endothelial and proximal

tubular cells more susceptible to transition metal induced toxicity. HUVECs and PTECs were equally susceptible to Zn²⁺, while for Fe³⁺ PTECs were more resistant as compared to HUVECs. Secondly, carnosine is able to prevent iron mediated toxicity in both cell types in a dose-dependent manner. The presence of serum carnosinase did not abrogate the protective effect of carnosine. Protection by carnosine was likely mediated by chelation of iron as it only occurred when carnosine was present during the iron challenge. Moreover,

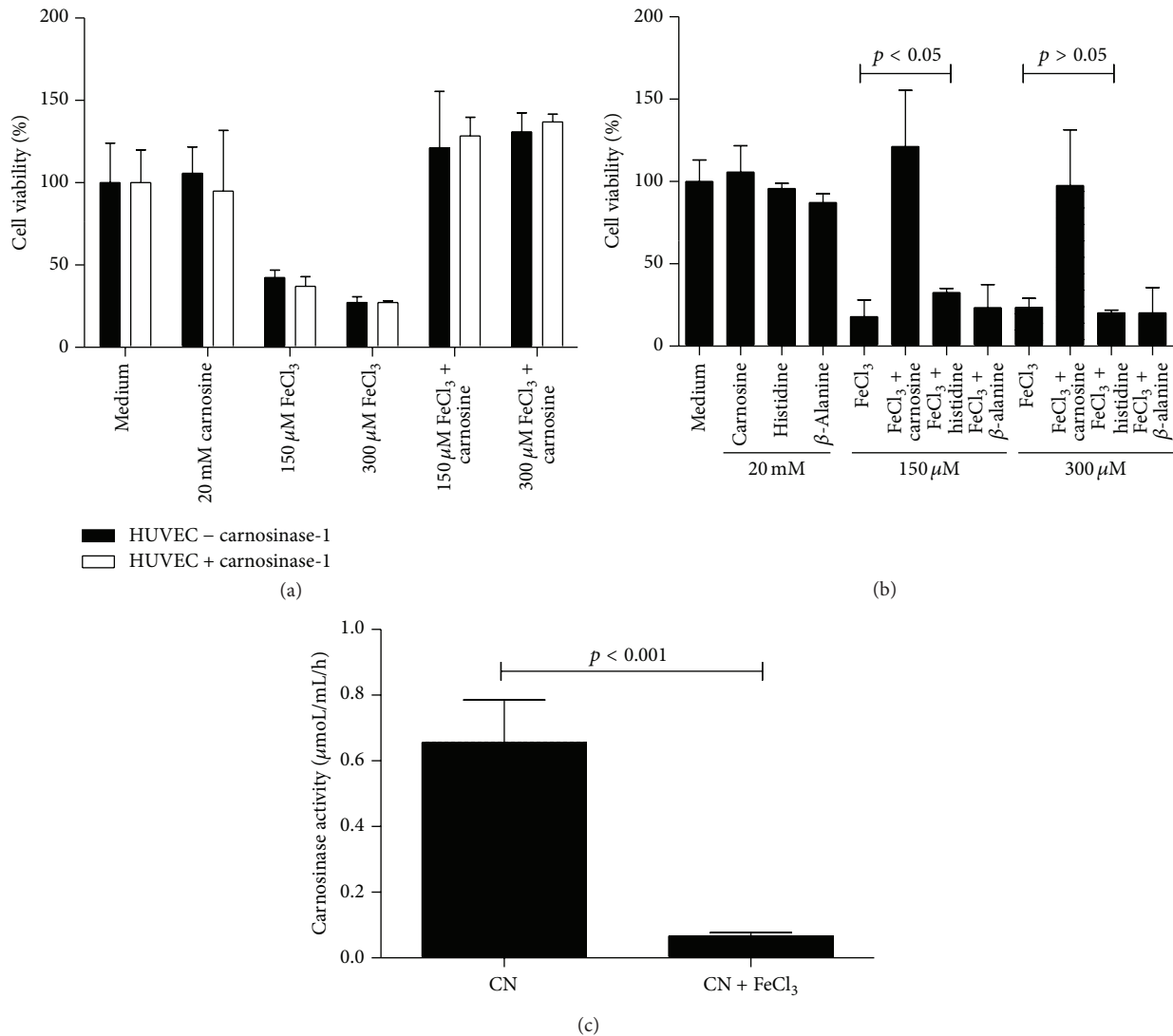


FIGURE 3: Ectopic expression of the human serum carnosinase-1 in HUVECs did not abrogate the protective effect of carnosine (a). The ability of the constitutive amino acids of carnosine, that is, β -alanine and L-histidine, to protect HUVECs against iron mediated toxicity was tested (b). Although L-histidine was slightly protective when iron concentration was 150 μ M, protection was clearly less compared to L-carnosine. No protection was afforded by β -alanine. Recombinant carnosinase activity was attenuated by the presence of 300 μ M $FeCl_3$ (c). CN: carnosine.

TABLE 1: TFRC expression under different conditions in either HUVECs or PTECs.

TFRC expression %	HUVEC	PTEC
Med	69.2 \pm 4.6	30.1 \pm 3.7
CN	69.8 \pm 3.4	16.5 \pm 1.7
Fe (60 μ M)	54.0 \pm 3.9	23.0 \pm 4.7
Fe (60 μ M) + CN	70.0 \pm 1.0	15.8 \pm 3.6

also deferoxamine was able to prevent iron mediated toxicity. Thirdly, it seems that both iron and carnosine were able to downregulate DMT1 and TFRC mRNA although at the protein level this was only observed for TFRC.

It has been suggested that dietary glycoxidation and lipoxidation products, rich in meat and other animal products, may at large be responsible for the increased risk of T2DM [15, 37, 38]. Glycoxidation and lipoxidation products, which occur after reaction between carbonyl groups on sugars and amine groups on proteins, DNA, and lipoproteins, are abundantly present in diabetic patients and are believed to be instrumental for diabetic complications. It is conceivable that glycoxidative and lipoxidative stress may act in concert with ROS to amplify tissue damage [39, 40]. Our data however do not indicate that HG makes endothelial and proximal tubular cells more susceptible to iron mediated toxicity. It can be argued that this might be a consequence of a relative short exposure to HG; however other studies using similar cells

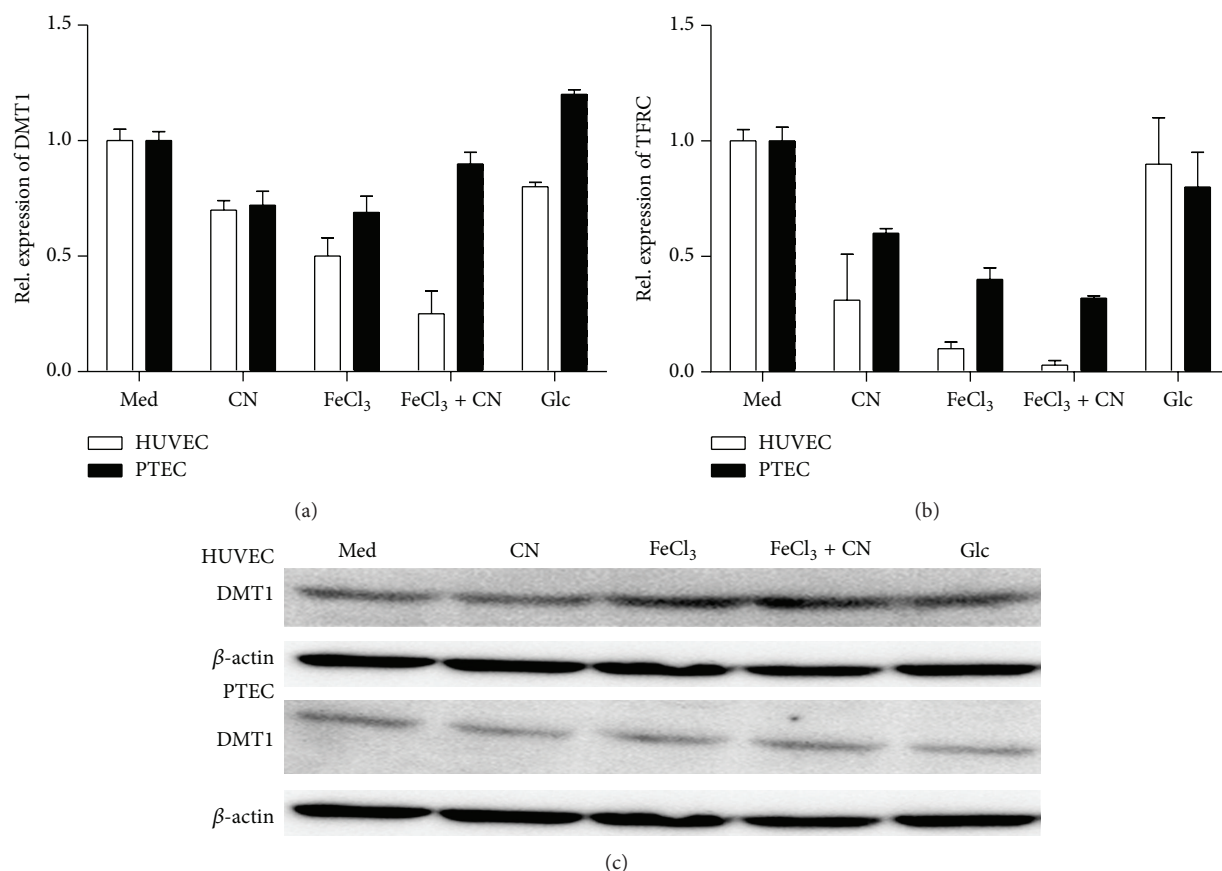


FIGURE 4: Influence of glucose and L-carnosine on DMT1 (a) and TFRC (b) mRNA expression. HUVECs (open bars) and PTECs (filled bars) were cultured for 24 h in the presence of 20 mM L-carnosine (CN), 60 μ M of FeCl₃, or both combinations. In addition cells were cultured for 3 days in HG medium (Glc). Total RNA was isolated and DMT1 (a) and TFRC (b) mRNA expression were assessed by qPCR. In (c) protein expression of DMT1 in HUVECs and PTECs is shown.

and culture conditions have demonstrated lipid peroxidation already after 48 h [41].

In plasma iron is linked in its ferric form to transferrin. The transferrin bound iron is subsequently taken up by cells through TFRC via an endocytic process. In the endocytic vesicle ferric iron is reduced and transferred to the cytosol via DMT1 [42, 43]. The expression of both TFRC and DMT1 was lower in PTECs as compared to HUVECs which may explain why PTECs were more resistant to iron. Yet it should be underscored that susceptibility of HUVECs to iron was not influenced by the presence of transferrin. This argues against the notion that intracellular iron accumulation via iron-transferrin mediated endocytosis was a major cause of toxicity in our *in vitro* model, albeit that it does not exclude the role of intracellular iron accumulation *per se* for toxicity.

Our data indicate that the protective effect of carnosine likely resides outside the cell. In support of this assumption is the observation that carnosine can only protect against iron mediated toxicity when it is present during the iron challenge. Previously the ferroxidase-like activity of carnosine has been linked to its cytoprotective effect [44]. Since ferroxidase catalyzes the oxidation of Fe²⁺ to Fe³⁺ ferroxidase activity indeed would have the potential to be cell protective as Fe²⁺

may generate damaging hydroxyl radical in the presence of H₂O₂. Yet in our study iron toxicity was performed with both Fe²⁺ (data not shown) and Fe³⁺ which in essence were toxic for HUVECs to a similar extent and carnosine was able to prevent toxicity mediated by both iron ions. Nonetheless, since we did not measure ferrous and ferric ions it cannot be excluded that carnosine pushes the equilibrium towards Fe³⁺.

It has also been suggested that complex formation of carnosine with transition metals underlies in part the antioxidative properties of carnosine, albeit that this has been questioned by others [45]. Complex formation has mostly been studied for Zn²⁺ and Cu²⁺ and is mainly attributed to the imidazole moiety within histidine [46, 47]. Like carnosine also DFO was protective, making iron chelation as an alternative explanation for the protective effect of carnosine. Overexpression of serum carnosinase however did not abrogate the protective effect of carnosine, which was explained by the finding that the carnosinase activity was significantly reduced in the presence of iron.

In conclusion our *in vitro* studies clearly demonstrate the ability of carnosine to ameliorate iron mediated toxicity in cultured endothelial and proximal tubular cells. In keeping with the high serum carnosinase concentration in humans,

the *in vivo* relevance of our findings needs to be addressed in future studies. The use of carnosine analogues or transition metal ions complexed carnosine that are resistant to hydrolysis by carnosinase may overcome this hurdle. The recent finding that the carnosinase-resistant, D-carnosine, or its bioavailable prodrug D-carnosine octylester has a salutary effect on lipoxidation mediated cellular injury in experimental atherosclerosis and renal disease is promising [48]. Yet it remains to be assessed if such compounds are also able to prevent iron mediated toxicity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Shiqi Zhang and E. Ntasis are equally contributing first authors.

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